Role of Schwann cell-derived Exosomes in Cisplatin-induced Hyperalgesia

A THESIS
SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY:

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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May 2018

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Acknowledgments

First and foremost, I would like to thank my advisor, Dr. Don Simone, for his continuous guidance and mentoring throughout my graduate studies. He has encouraged me to become an independent scientist, and I have learned much from working in his lab. I am grateful for each and every member of the Simone lab for their kind support and thoughtful conversations: Megan Uhelski, Samuel Erb, Iryna Khasabova, Sergey Khasabov, Malcolm Johns, Sarah Shueb, and Rebecca Speltz. Special thanks to Dr. Iryna Khasabova for her dedicated help in the development of this thesis and for teaching of various research techniques. It would not have been possible to accomplish this study without her insightful feedback.

I would also like to thank Dr. Julie Olson, and Nhungoc (Ti) Luong for their technical assistance in immunohistochemistry, as well as Dr. Mary Lunzer for helping with the intrathecal injections. A portion of this work was carried out in the Minnesota Nano Center which receives partial support from the National Science Foundation through the NNCI program. I would like to express my gratitude to Dr. James Marti for his assistance with the nanoparticle tracking analysis. I would also like to thank Dr. Yingchun Zhao at Mass Spectrometry Core Facility, Masonic Cancer Center, University of Minnesota for his wonderful technical work in MS analysis.

I would like to thank Ann Hagen for all of her administrative assistance, as well as a tremendous amount of emotional support, in the Graduate Program in Oral Biology. I

would like to thank Dr. Mark Herzberg and the MinnCResT program for all the research support, feedback and training.

This work was supported by NIH grant HL135895 and MinnCResT NIDCR grant T90 DE022732. The content is solely the responsibility of the author and does not necessarily represent the official views of the NIH or the NIDCR.

Last, but not least, I would like to express my deepest gratitude and love to my parents and my sister Junie for their never-ending love and support in completing my thesis work. Without them, none of this would have been possible.

To my family

Abstract

Painful peripheral neuropathy is a common dose-limiting side effect associated with cisplatin treatment. Cisplatin is unable to cross the blood-brain barrier, and its neurotoxicity is limited to the peripheral nervous system (PNS). In the PNS, Schwann cells are an essential component supporting dorsal root ganglion (DRG) neuron viability, and impairments in Schwann cell biology contribute to cisplatin-induced painful neuropathy. We explored the role of Schwann cell-derived exosomes in the development of cisplatin-induced hyperalgesia. Consistent with our previous reports, daily injection of cisplatin (1 mg/kg, i.p.) for 7 days produced mechanical hyperalgesia in C3H/HeN mice. To investigate the impact of exosome signaling in the development of cisplatin-induced hyperalgesia, exosomes isolated from the sciatic nerves of cisplatin-treated mice were injected intrathecally into naïve mice for 5 consecutive days (7 μg of total protein/10 μl, i.t.). Mechanical hyperalgesia was observed after the second injection of exosomes, mimicking the effect of cisplatin alone and supporting the involvement of integrated exosome signaling in hyperalgesia produced by cisplatin. Intrathecal administration of Schwann cell-derived exosomes activated microglia, and analysis of exosomal content indicated mediators of neuronal sensitization at the central level. Collectively, our results indicate that Schwann cells affected by cisplatin contribute to mechanical hyperalgesia and exosomes are an important signaling mediator for glia-neuronal communication.

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1. Introduction

More than one million new cases of cancer occur in the United States each year (American Cancer Society, 2017). Cisplatin is a platinum-derived chemotherapeutic drug that continues to be one of the most effective and widely used of anticancer drugs in the clinics (Florea and Busselberg, 2011). However, the use of cisplatin is associated with unwanted side effects, including painful peripheral neuropathy that is a dose-limiting factor affecting survival. More than 50% of leukemia, lymphoma, colorectal- and breast cancer patients develop some degree of chemotherapy-induced peripheral neuropathy, and the prevalence of cisplatin-induced peripheral neuropathy is as high as 57% in cancer survivors (Streckmann et al., 2018; Travis et al., 2014).

Cisplatin does not cross the blood-brain barrier but accumulates in the dorsal root ganglia (DRGs) and peripheral nerves (Gregg et al., 1992). Hyperalgesia results from cisplatin-induced neurotoxicity to DRG neurons responsible for transmitting pain (Grisgold et al., 2012). Cisplatin produces cytotoxicity when platinum-DNA adducts are formed and compromises nuclear and mitochondrial DNA (mtDNA) transcription. This causes DNA damage and subsequently induces apoptosis in cancer cells (Dasari and Tchounwou, 2014). Research shows that cisplatin-evoked mtDNA damage causes functional changes within the mitochondria of DRG neuronal cells (Podratz et al., 2011). Mitochondrial function is mandatory for the maintenance of cellular respiration, regulation of intracellular calcium levels, and protection from oxidative stress via the regulation of reactive oxygen species (ROS) accumulation (Carrozzi et al., 2015). When ROS

increases, oxidative stress is induced and damages cellular functions, leading to apoptosis (Martindale and Holbrook, 2002; Dasari and Tchounwou, 2014). However, the intercellular mechanisms, particularly within the glial-neuron interaction, by which cisplatin-induced neuropathic pain occurs is yet unknown.

Mechanisms underlying cisplatin-induced hyperalgesia include the activation and interaction of glial cells and neurons (Tsuda, 2017). Among these glial cells are Schwann cells (SCs) and microglia. Microglia are a type of glial cell that function as the immune cells of the central nervous system (CNS) (Prinz and Priller, 2014). Microglia release pro-inflammatory cytokines, including TNF-α, which sensitize nociceptive dorsal horn neurons and cause central sensitization and hyperalgesia (Berta et al., 2014). Microglia also act as phagocytes that clear up injured cells and debris (Kuno et al., 2005). Following injury, microglia are activated and engaged in both innate and adaptive immune response of the CNS. In particular, microglia of the spinal cord have been shown to be key players of CNS immune response during neuropathic pain (Olson, 2010).

Schwann cells (SCs) are a type of peripheral glial cell that play a crucial role in maintaining the structure and function of both myelinated and unmyelinated peripheral axons whose cell bodies are located in the DRGs (Lopez-Verrilli et al., 2013). SCs are involved in axonal regeneration, neuroprotection, and neuro-immune interactions and affect neuronal survival (Quintes et al., 2010; Fields and Stevens-Graham, 2002; Glenn and Talbot, 2013). Whether cisplatin-evoked damage of SCs alter normal interactions

between SCs and DRG neurons, and thereby contribute to cisplatin-induced hyperalgesia, remains unclear. The cytotoxicity of cisplatin treatment affects SCs as they are post-mitotic cells residing outside of the blood-brain barrier. Given the role of SCs in myelination of peripheral nerves, the malfunctioning of SCs may play a part in neuronal sensitization (Lopez-Verrilli et al., 2013). With cisplatin treatment, lack of myelination and support by SCs may cause quicker deterioration of myelinated nerves, or the release of pro-apoptotic signaling may facilitate irreversible degeneration of nearby non-myelinated nerves.

Of the several mechanisms implicated in glia-neuron communication, such as Calcium signaling or gliotransmitter release, one key player suggested to be the mediator in the pathogenesis of inflammation and neurodegeneration is exosomes (Araque and Navarrete, 2010; Gupta and Pulliam, 2014). Exosomes are nanovesicles, ranging from 30-100 nm in size, that carry various signaling molecules including proteins, mRNA, and miRNA from host to target cells. Exosomes are generated by inward budding of the endosomal limiting membrane and stored in multivesicular bodies (MVBs) before release (Colombo et al., 2014). They are released into the extracellular space by fusion of MVBs with the plasma membrane (Fröhlich et al., 2014). In the CNS, exosomes have been implicated in neurodegenerative diseases (Kalani et al., 2013). It has also been shown that astrocyte-derived exosomes may have a role in disease spreading and motor neuron pathology. Direct involvement of exosomes in contributing to oxidative stress has been demonstrated in the interplay between motor neurons and glial cells using a murine

model of Amyotrophic lateral sclerosis disease, where astrocyte-derived exosomes were shown to efficiently transfer mutant SOD1 to spinal neurons and induce selective motor neuron death (Basso et al., 2013). Importantly, oxidative stress is an important factor related to neuropathic pain following nerve injury (Kuo et al., 2017), as well as in disease states including sickle cell disease (Tran et al., 2017) and chemotherapy-induced peripheral neuropathy (Joseph and Levine, 2009; Xiao et al., 2011). Also, macrophage-derived exosomes have been shown to release cytokines and miRNAs that mediate inflammation and pain in both innate and adaptive immune pathways (McDonald et al., 2014) Exosomes secreted by oligodendrocytes have been shown to enter neurons to make their cargo functionally available to the neuronal metabolism (Fruhbeis et al., 2013). Similarly, in the peripheral nervous system, SC-derived exosomes are known to be involved in peripheral nerve regeneration (Lopez-Verrilli et al., 2013). Exosomes secreted by SCs represent one potential avenue by which SCs interact with DRG neurons, and SC-derived exosomes from cisplatin-damaged SCs may contribute to cisplatin-induced neurotoxicity and hyperalgesia. Specifically, exosomes from SCs following cisplatin treatment may carry mediators that facilitate sensitization of peripheral nociceptive neurons and activation of microglia in the spinal cord.

In the present study, we investigated whether SC-derived exosomes were involved in cisplatin-evoked hyperalgesia by examining behavioral changes induced in naïve mice when SC-derived exosomes from cisplatin-treated mice are administered intrathecally. We then identified molecules within these exosomes that may contribute to pro-

inflammatory signaling that can cause neuronal sensitization and hyperalgesia, and the potential activation of microglia following exosome administration as a potential mediator for the underlying central sensitization.

It must be clarified that exosomes identified as SC-derived exosomes within this study are exosomes isolated from the whole sciatic nerve. These exosomes isolated from the whole sciatic nerve contain exosomes from SCs but also others, including satellite cells and neurons. Unfortunately, SCs are difficult to culture and exosomes from SC culture is even more limited in quantity following cisplatin treatment. In order to verify that exosomes from SCs alone have same hyperalgesic effect, preliminary data was collected using exosomes from SC culture. Hyperalgesia was observed in naïve mice injected with exosomes from cisplatin-treated SCs (data included in Figure 4A).

2. Materials and Methods

2.1 Animals

Adult male C3H/HeJ mice (Jackson Laboratories), 2 to 4 months of age were used. Mice were housed in cages of three to four mice per cage, maintained on a 12-h light-dark cycle, and had free access to food and water. Behavioral testing occurred between 8:00 AM and 5:00 PM. All protocols and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.1 Drug Treatment

The platinum chemotherapy agent cis-dichlorodiammine platinum(II) (cisplatin; LKT Laboratories) was prepared in a 1 mg/ml solution in normal saline and administered intraperitoneally at 1 ml/kg.

2.3 Behavioral Assessment of Mechanical Hyperalgesia

Withdrawal response frequency evoked by a standard calibrated von Frey monofilament was used to assess the development of mechanical hyperalgesia. Baseline measurements were taken prior to drug injection to acclimate the mice to the experimental testing environment and to evaluate initial level of response prior to any injections. Mice were placed under isolated glass containers on an elevated mesh platform and were given at least 30 minutes to acclimate prior to testing. Withdrawal responses of hind paw was assessed using a von Frey monofilament of 0.4g (3.9mN). The monofilament was applied

to the plantar surface of each hind-paw for approximately 2 s and the withdrawal response was indicated by the rapid removal of the paw and was occasionally followed by brief flinching and/or licking of the paw. The monofilament was applied ten times with an inter-stimulus interval of approximately 5 s, and the total number of withdrawal responses were recorded. For cisplatin-treated mice from which exosomes were isolated, only subjects exhibiting consistent mechanical allodynia (withdrawal frequencies of at least 60%) in both hind paws were used. The experimenter was blind to the treatment conditions.

2.4 Exosome Isolation and Administration

The protocol provided from Invitrogen™ was used as a reference using the Total Exosome Isolation reagent (4478359). Samples were prepared by mashing the extracted sciatic nerves using a 10-mL syringe plunger against a sterile mesh inside a petri dish. The mashed exosomes were re-suspended in complete DMEM media, and more media was added (if needed) to total the final volume to be 1000 μL. Samples were centrifuged for 10 minutes at 1200 rpm. Supernatant was transferred to 1.5-mL micro-centrifuge tubes. 500μL of Total Exosome Isolation reagent was added (1:2 ratio per suggested protocol). Culture media/reagent mixture was vortexed to ensure thorough mixture and incubated at 4°C overnight. After overnight incubation, samples were centrifuged at 10,000 rcf for 1 hour. Supernatant was removed, and exosomes were suspended in PBS and stored at 4°C until use. Exosomes were aliquoted at 7 μg of protein per 10 μL and

each 10 μL were administrated intrathecally to naïve mice for five consecutive days.

2.5. Intrathecal injection

Intrathecal injections were delivered by spinal cord puncture. Spinal cord puncture was made with a 30 G needle between the L5 and L6 to deliver 10 μL of exosomes suspended in saline to the cerebral spinal fluid. All drugs were administered to conscious mice according to the method of Hylden and Wilcox (1980) for i.t. injections. Mice were observed in their home cages at least 15 min following injection to monitor potential occurrence of paralysis.

2.6 Nanoparticle Tracking Analysis

Particle size distribution of exosomes isolated from the sciatic nerves was analyzed in order to verify that the re-suspended exosomes are in the exosome size range of 30 to 100 nm. Particle size distributions were determined using the Nanosight LM-10 (Malvern Instruments, Malvern UK), based on the technique of nanoparticle tracking analysis (NTA). The NTA process directs intense laser light at particles suspended in a sample cell. The particles undergo Brownian motion in the suspending fluid, with the smaller lighter particles moving more quickly than the larger, more massive particles. The light scattered by these particles is collected by a microscope objective and directed to a very sensitive CMOS camera, which records video of the moving particles at 30 frames per second. Image analysis software is used to track the motion of each particle over several seconds, then calculates a diffusion coefficient D of each particle based on its recorded

motion. These diffusion coefficients can then be used calculate particle sizes, using the Stokes-Einstein equation:

$$D = \frac{RT}{N_A}\frac{1}{6\pi\eta a}$$

where R is the universal gas constant, T the absolute temperature, N_A is Avogadro's number, η is the dynamic viscosity of the suspending fluid (water in this case), and a is the particle's hydrodynamic diameter. The Nanosight calculates particle diameters for 10^3 to 10^4 particles per sample and outputs a number-weighted size distribution.

2.7 Western Blot

In order to have a secondary confirmation of successful exosome isolation, western blotting was performed to confirm the presence of CD-63, a primary surface protein marker of exosomes (Choi et al., 2012; Menay et al., 2017). Western blotting of exosomes was performed on 4-20% precast polyacrylamide gel Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system for 1 h. 30 μg of protein was loaded on each lane, and the proteins were transferred to a PVDF membrane after running SDS-PAGE. Transferred membranes were blocked for 1 h, CD-63 primary antibody was applied at 1:500, and the blots were incubated overnight at 4 °C. The blots were washed three times (10 min each) with 10 mL TBS/T and were incubated with secondary antibody (1:15,000) on a shaker for 1 h at room temperature. Then, the blots were washed three times with TBS/T and imaged.

2.7 Immunohistochemistry

Spinal cords and DRGs were isolated from treated mice on post-injection day (PID) 7, and tissues were fixed in 4% paraformaldehyde for 24 h. Tissues were transferred to 70% ethanol for another 24 h, then was paraffin embedded. Embedded tissues were sectioned with a microtome at 5 μm. Mounted sections were washed with xylene, then 100%, 95%, 70% and 50% ethanol, and incubated in citrate buffer overnight at 60 °C. Following day, the slides were blocked with blocking buffer (2% BSA in PBS + 0.05% Tween-20) for 1 h. Slides were washed then treated with anti-Iba-1 rabbit polyclonal antibody at 1:1000 (Wako, 019-19741) and incubated overnight at 4 °C. Following day, the slides were washed and incubated with biotinylated secondary antibody, then detected by Streptavidin HRP and TSA Plus Cyanine 3 system (PerkinElmer), and captured using a confocal microscope. Images were then exported to ImageJ software for quantitative analysis. Analysis of the immunofluorescent Iba-1+ cells was performed using a constant set of parameters (exposure time, gain and post-image processing) and the total area and mean integrated intensity of activated cells was compared between groups.

2.8 Schwann Cell culture

In order to identify the contents and role of exosomes derived specifically from Schwann cells, Schwann cell primary culture was used for mass spectrometry analysis. Sciatic nerves of naïve mice were isolated and incubated at 37 °C for 2 weeks in complete DMEM. After incubation, nerves were transferred to a fresh plate containing complete

DMEM with 0.125% Collagenase IV and 1.25u/mL Dispase I and incubated for 20 h at 37 °C. Nerves were dissociated with a Pasteur pipette, and centrifuged for 10 min at 1200 rpm. Cells were washed in melanocyte growth media and forskolin and centrifuged once more for 10 min at 1200 rpm and were carefully re-suspended on pre-coated plates. Cells were incubated at 37 °C with necessary media changes until confluent. Cells were treated with cisplatin (4μg/ml) for 48 h, which has been shown in previous literature to mimic the cytotoxic effect seen the *in vivo* treatment (Khasabova et al., 2012).

2.9 Mass Spectrometry

Mass spectrometry analysis was performed in order to identify the potential cargo of the exosomes and to identify possible pro-inflammatory or cytotoxic components that may be released from Schwann cells. In order to isolate exosomes released from Schwann cells only, Schwann cell culture was used and treated with cisplatin. Exosomes released from cisplatin-treated Schwann cells *in vitro* were isolated and protein levels were quantified. Proteins were run on the polyacrylamide gel and digested with trypsin. Digested proteins were subjected to a desalted, using UltraMicro spin tips. LC-MS/MS analyses were performed using an RSLCnano system (Dionex) and Orbitrap Elite Hybrid (Thermo Scientific) mass spectrometer. Each fraction was fractionated using a column (75 μm inner diameter, 35 cm) manufactured in-house and eluted at 300 nl/min using an 80-min gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. A data-dependent acquisition was performed set at 60,000 resolutions over 350–1500 m/z range. High-energy collision dissociation (HCD) fragmentation was carried out with 1.4 m/z

isolation window and normalized collision energy (NCE) of 40%. The maximum injection times were 100 and 500 ms, and ion targets were 10^6 and 5 x 10^4 for MS and MS/MS (at 15,000 m/z resolution) (Dator et al.,2017; Blanden et al., 2018). The .raw files were analyzed with MaxQuant version 1.6.0.16 and searched against the mouse proteome database. Data was exported to Microsoft Excel and peptides were sorted by coverage.

2.10 Data Analysis

Two-way analysis of variance (ANOVA) with repeated measure was used to compare the effects of daily administration of cisplatin, and i.t injections of exosomes (or vehicle) on withdrawal response frequency over time. Nanoparticle tracking analysis was reported as mean +/- standard deviation and mode +/- standard deviation. Western blot of CD-63 was quantified by comparison of expression relative to the control group. For images of activated immunofluorescent Iba-1+ cells, Student's t-test was used to compare and evaluate the statistical differences between groups.

3. Results

3.1 Mechanical Hyperalgesia produce by Cisplatin

Consistent with our previous reports (Khasabova et al., 2012; 2013), daily injection of cisplatin (1 mg/kg, i.p.) for 7 days produced mechanical hyperalgesia in C3H/HeN mice. Mechanical hyperalgesia developed by 24 h after the third injection with $p < 0.001$ compared to saline-treated mice as well as compared to baseline. Withdrawal response frequency for control mice remained near baseline for the duration of the treatment period (Fig. 1).

3.2 Confirmation of Sciatic nerve-derived Exosomes

Nanoparticle tracking analysis of exosomes re-suspended in PBS verified the successful isolation of exosomes with mode of 107.8 +/- 13.6 nm and mean of 167.8 +/- 4.5 nm (Fig. 2). Particle size suggests that majority of the injected content contains exosomes isolated from the sciatic nerves. We next verified the isolation of sciatic nerve-derived exosomes using a western blot for CD-63, a common surface protein found on exosomes (Choi et al., 2012; Menay et al., 2017) and identified the presence of CD-63 in exosomes isolated from the sciatic nerves of saline-treated mice, as well as exosomes from cisplatin-treated mice (Fig. 3).

3.3 Hyperalgesic Effect of Exosomes from Cisplatin-treated Mice

Five consecutive intrathecal injections of exosomes isolated from the sciatic nerves of cisplatin-treated mice induced mechanical hyperalgesia in naïve mice by 24 h after fourth

injection, with $p < 0.001$ compared to the naïve-exosome treated mice as well as compared to baseline (Fig. 4B). Withdrawal response frequencies for control mice remained near baseline for the duration of the treatment period. The hyperalgesic effect of exosomes isolated from sciatic nerves of cisplatin-treated mice mirrors the effect of systemic treatment of cisplatin alone.

3.4 Increased Activation of Microglia

Comparison of microglial activation with Ionized calcium binding adaptor molecule 1 (Iba-1) in the spinal cords of mice treated with cisplatin-exosomes showed upregulation and greater activation of microglia than compared to the control-exosomes (Fig. 5). The increase in mean integrated intensity of microglia in the treated conditions was statistically significant with $p < 0.005$ compared to control, with 88% increase in the total area of activated glia.

3.5 Potential contents of Exosomes from Cisplatin-treated Mice

Mass spectrometry and proteome analysis of Schwann cell-derived exosomes under cisplatin and control conditions indicate the presence of proteins involved in the TNF-α pro-apoptotic pathway (data not shown). Proteins involved in inflammatory response, including annexin and perilipin, were present in the exosomes released from SCs treated with cisplatin. Lack of ATP synthase, a protein regulating mitochondrial function, was also noted in the exosomes released from SCs treated with cisplatin when compared to the non-treated SC exosomes.

4. Discussion

The present study showed that cisplatin treatment causes mechanical hyperalgesia and that this occurs, at least in part, by SC-derived exosomes that may contribute to neuronal sensitization during cisplatin-induced peripheral neuropathy. Seven daily injections of cisplatin administered systemically into naïve mice resulted in increased withdrawal frequency response of the hind paw to the von Frey stimulus of 0.4 g, confirming the development of mechanical hyperalgesia, consistent with our previous reports (Khasabova et al., 2012; Uhelski et al., 2015). This increase in withdrawal frequency was over 60% by the end of the 7-day treatment (from a baseline of less than 20%), and was statistically significant compared to baseline, as well as to the control group. The withdrawal frequency response of the saline-treated mice remained near baseline throughout the 7-day period (Fig. 1).

As a general rule, two different methods of exosome characterization are necessary to confirm successful isolation of exosomes (Tang et al., 2017). In this study, the isolation of exosomes from sciatic nerve was confirmed by nanoparticle tracking analysis and western blotting. Size distribution of exosomes agreed with the designated size range of exosomes, with mean of 167.8 +/- 4.5 nm and mode of 107.8 +/- 13.6 nm (Fig. 2). From our analysis of the nanoparticles, it is possible that amongst the exosomes there may be microvesicles, apoptotic bodies, and other debris that may be responsible for particles detected at larger size ranges (Willms et al., 2016). This amount of variability in size is

consistent with previous studies on exosomes (Alvarez et al., 2012; Colombo et al., 2014). One common criticism of this methodology is that it utilizes light scattering to rapidly calculate the total number and size distribution of samples. Unfortunately, it does not distinguish between vesicles, protein aggregates, or debris, and may over-estimate the quantity of exosomes (Gercel-Taylor et al, 2012; Helwa et al., 2017; Tang et al., 2017). To address this issue, we standardized the amount of exosomes injected using protein quantification. Proteins were quantified using a Pierce BCA Protein Assay (Thermo Fisher), and mice were injected with exosomes at 7μg of protein each day. The second characterization of exosomes was performed via western blotting. Once again, the total protein loading was used as an internal control. The results showed vivid expression of exosome-enriched protein CD-63 (Fig. 3). CD-63 is a transmembrane tetraspanin protein characteristic of exosomes and is commonly used to identify exosomes (Choi et al., 2012; Menay et al., 2017; Lankford et al., 2018; Willms et al., 2016).

The isolated exosomes were injected intrathecally into naïve mice to observe changes in mechanical hyperalgesia. This route of administration was chosen in order to deliver exosomes as close to the peripheral nerve as possible so that direct effect on neuronal sensitization could be studied. Because of the size of the mouse paw, we were concerned about tissue damage from repeated intraplantar injections. The limitation of this method is that multiple intrathecal injection could induce paralysis in hind legs of mice. Mice that become paralyzed (n= 3) were excluded from the study because withdrawal responses to mechanical stimuli could not be properly assessed. Another caveat of intrathecal

administration is that it is difficult to isolate the peripheral effect of neuronal sensitization. Since the exosomes are being delivered to the CSF, a greater involvement of the CNS may be induced, leading to downstream peripheral nerve damage. In order to observe any acute peripheral effect of exosomes on nerves, exosomes were administered directly into the hind paw via a single intraplantar injection, and this did not produce hyperalgesia. It is difficult to identify whether it is the amount of exosomes or the time window tested that resulted in no acute hyperalgesic effect. Unfortunately, multi-day intraplantar injections to mice is not feasible, and exosomes were therefore delivered intrathecally.

Our results show that intrathecal injections of exosomes isolated from the sciatic nerve of cisplatin treated mice for five consecutive days of resulted in the development of mechanical hyperalgesia. These mice exhibited an increase in withdrawal frequency by 24 h after the 4^{th} intrathecal injection, with continuous increase in withdrawal frequency in the following days (Fig. 4B). This response was statistically significant when compared to baseline, as well as to the control group. The withdrawal frequency response of the mice treated with exosomes isolated from saline-treated mice had no changes in behavior. This behavioral change mirrors the effect of systemic injection of cisplatin treatment. Since exosomes can freely cross the blood brain barrier, intravenous administration of exosomes may produce similar changes in behavior. Although the isolation of exosomes is very limited, a dose response of exosomes isolated from the sciatic nerve of cisplatin-treated mice may be conducted in the future. Our results suggest

that exosomes play an important role in communication between glia and neurons to induce neuronal sensitization. This agrees with previous studies where neuronal exosomes have been shown to activate microglia to promote activity-dependent pruning, and oligodendroglial exosomes mediate antigen transfer to dendritic cells (Bahrini et al., 2015; Frubeis et al., 2012). For our study, exosomal activation of microglia will help identify whether SC-derived exosomes induce an immune response during transfer of cytotoxic contents to nearby neurons.

We hypothesized that these exosomes from SCs contribute to the hyperalgesia. However, exosomes isolated from the whole sciatic nerve contains other host cells from which some of these exosomes may be derived, including satellite cells and neurons. Therefore, the behavioral results of this study do not isolate the role of SC-derived exosomes. In order to verify the pro-inflammatory role of SC derived exosomes, we used SC culture to determine the contents of exosome cargo. Primary SC culture was treated with cisplatin 13μM for 48 h, as this concentration and duration was tested to induce cytotoxicity *in vitro*, without completely killing all cells. Exosomes were collected from media and subjected to mass spectrometry analysis.

Mass spectrometry analysis suggests multiple pro-inflammatory and pro-apoptotic proteins, as well as vesicle-related proteins, to be found in Schwann cell-derived exosomes following cisplatin treatment. Some of the most notable proteins were: tumor necrosis factor (TNF) ligand, NF-kappa-B inhibitor-interacting Ras-like protein, and

Amyloid β precursor protein. Tumor necrosis factor alpha is an inflammatory cytokine released by activated glia and is involved in neuronal sensitization (Lewitus et al., 2016). NF-kappa-B is known to induce pro-inflammatory signaling cascade upon injury or infection (Lawrence et al., 2009). Specifically, macrophage-derived exosomes from lipopolysaccharide-stimulated cells has been shown to induce NF-kappa-B activation in naïve cells (McDonald et al., 2014). Amyloid β is a toxic protein aggregate responsible for the development of Alzheimer's disease and has been shown to get effectively packaged into exosomes to induce a pro-inflammatory cascade (Gupta and Pulliam, 2014; Murphy and LeVine, 2010; Rajendran et al., 2006). Results of the mass spectrometry analysis agrees with previous studies showing that cisplatin activates NF-kappa-B pathway, contributing to cisplatin resistance as well as in inducing mechanical hyperalgesia (Lagunas et al., 2008; Park et al., 2014).

Given these results, it appears that cisplatin treatment results in activation of glial cells. Using a primary culture of SCs gives us the confidence that SCs are damaged by cisplatin, and that during this process, SCs release exosomes that carry pro-inflammatory and pro-apoptotic signaling molecules. It is necessary to perform these behavioral experiments using these exosomes treated in with cisplatin in culture. Preliminary assessment of exosomes from Schwann cell culture has similar effect (data not shown due to small sample size). By the analysis of exosomes derived from SCs *in vitro*, we are able to conclude that amongst many potential mechanisms that SCs are affected by cisplatin and that SC-derived exosomes contribute to mechanical hyperalgesia via pro-

inflammatory and pro-apoptotic signaling.

Glia play an important role in inflammation in the CNS and specifically have been noted to be involved in the development of peripheral neuropathy (Ji et al., 2013). The suggestion of glial activation following cisplatin treatment prompted us to look at microglial activation in the spinal cords of the treated mice. Microglial activation marker Iba-1 was used, as it is a microglia and macrophage specific calcium-binding protein, present on activated microglia. Compared to the spinal cords of control mice, a notable increase in microglial activation was observed in mice injected with exosomes isolated from the sciatic nerves of cisplatin-treated mice. Activation of microglia suggests central sensitization induced by intrathecal administration of exosomes. Given the intrathecal route of exosome administration, it is unclear whether SC-derived exosomes will induce increased microglial recruitment and activation under biological conditions when mice are treated with cisplatin. Although it is difficult to isolate central and peripheral effects on neurons with an intrathecal administration of exosomes, the results of this study demonstrate that SC-derived exosomes contribute to neuronal sensitization at the central level by recruitment and activation of microglia.

Exosomes are known to carry mRNA miRNA in their cargo, and these contents reflect the origin of host cells (Kowal et al., 2014; Santangelo et al., 2017; Vlassov et al, 2012). Exosomes have been shown to release miRNA from viruses upon infection and spread viral miRNA to monocytes (Pegtel et al., 2010). Particularly, under pathological

conditions, miRNAs with pro-inflammtory functions have been shown to be found in exosomes (De Toro et al., 2015; Guo and Guo, 2015). These studies suggest that future analysis of mRNA and miRNA may provide further insight to the pro-inflammatory nature of exosomes and mechanisms by which they produce neuronal sensitization and pain.

A better understanding of glia-neuron communication, and specifically the interaction of SCs and peripheral neurons will provide further insight in addressing chemotherapy-induced neuropathic pain. The understanding of SC-neuron interaction in evoking hyperalgesia can potentially provide new avenues of treatment for reducing neurotoxicity and neuropathic pain from chemotherapy. Furthermore, greater insight on neural mechanisms underlying cisplatin-induced peripheral neuropathy, specifically the role of exosomes in the generation and maintenance of pain, can be extended to understand the role of exosomes in other types of chronic pain. An understanding of contributions of SCs and their exosomes may provide a greater understanding of the mechanism underlying painful peripheral neuropathy that decreases efficacy of cisplatin treatment.

5. Conclusion

In conclusion, this study demonstrates that SC-derived exosomes contribute to cisplatin-induced mechanical hyperalgesia by the releasing pro-inflammatory and cytotoxic cargo to nearby neurons. A better understanding of the mechanisms underlying cisplatin-induced peripheral neuropathy can provide potential ways of addressing and minimizing this painful side effect of chemotherapy treatment. Furthermore, the results of this study suggest exosomes to be an essential route of intracellular communication in the pain signaling pathway and may provide novel targets for the development of new pharmacological agents that prevent cisplatin-induced neuropathic pain.

Figure Legends

Figure 1. Cisplatin administration was associated with increased hind paw withdrawal frequencies relative to both baseline assessments and saline-treated mice starting on post-injection day 3. Withdrawal responses in saline controls remained near baseline throughout the treatment period. *P < .001 vs. saline; #P < .001 vs. baseline.

Figure 2. Size analysis of exosomes isolated from the sciatic nerves of cisplatin-treated mice show presence of exosomes with mode of 107.8 +/- 13.6 nm and mean of 167.8 +/- 4.5 nm.

Figure 3. Exosomes were subjected to SDS-Page and western blot, using a common exosome surface protein marker, CD-63, and the western blot bands were quantified and normalized to control. Both exosomes isolated from sciatic nerves of saline-treated mice (lane 1) and cisplatin-treated mice (lane 2) show presence of CD-63.

Figure 4. A) Administration of exosomes isolated from cisplatin-treated Schwann cells was associated with increased hind paw withdrawal frequencies relative to exosomes isolated from naïve Schwan cell culture (n= 2). B) Administration of exosomes isolated from cisplatin-treated mice was associated with increased hind paw withdrawal frequencies relative to both baseline assessments and saline-treated mice starting on post-injection day 4. Withdrawal responses in controls remained near baseline throughout the treatment period. *P < .001 vs. saline; #P < .001 vs. baseline.

Figure 5. Staining of microglia show upregulation and activation of microglia in lumbar spinal cord sections of mice injected with exosomes from cisplatin-treated mice (B,D) compared to control (A,C). Red indicates Iba+ microglia, and blue indicates DAPI.

Figure 1.

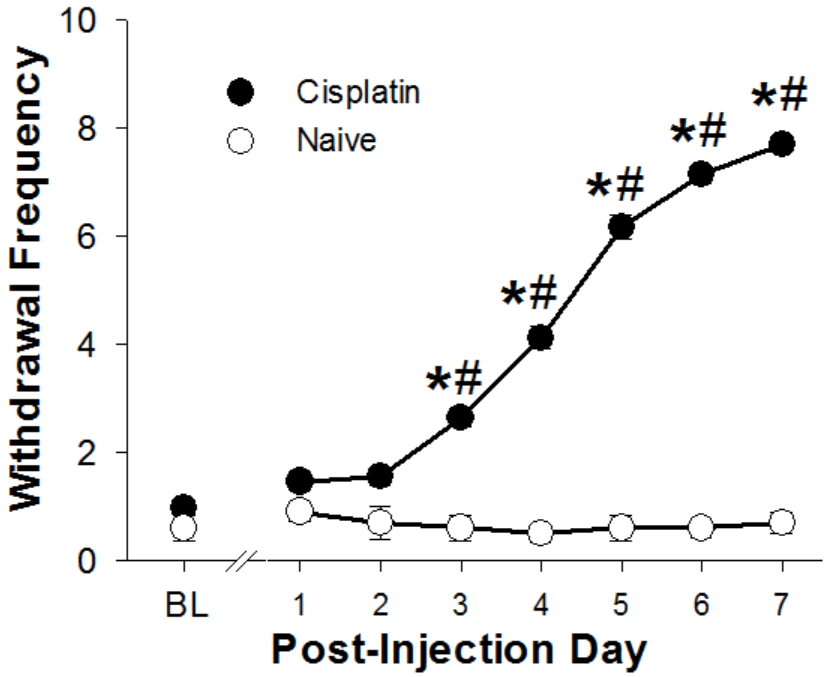

Withdrawal Frequency
10
8
6
4
2
0
Cisplatin
Naive
*#
*#
*#
*#
*#
BL
1
2
3
4
5
6
7
Post-Injection Day

Figure 2.

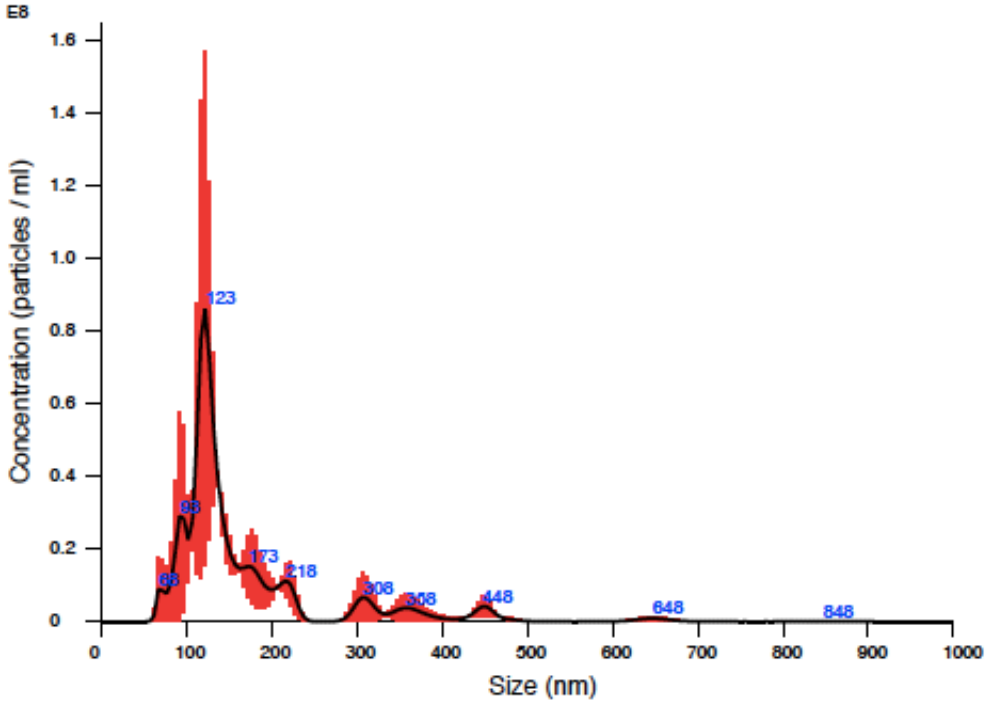

E8
Concentration (particles / ml)
Size (nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
700
800
900
1000
123
88
66
173
218
308
368
448
648
848

Figure 3.

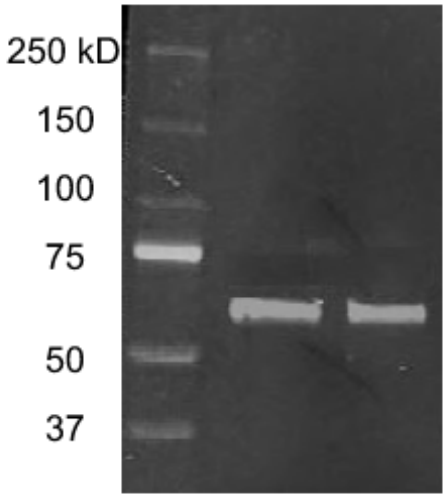
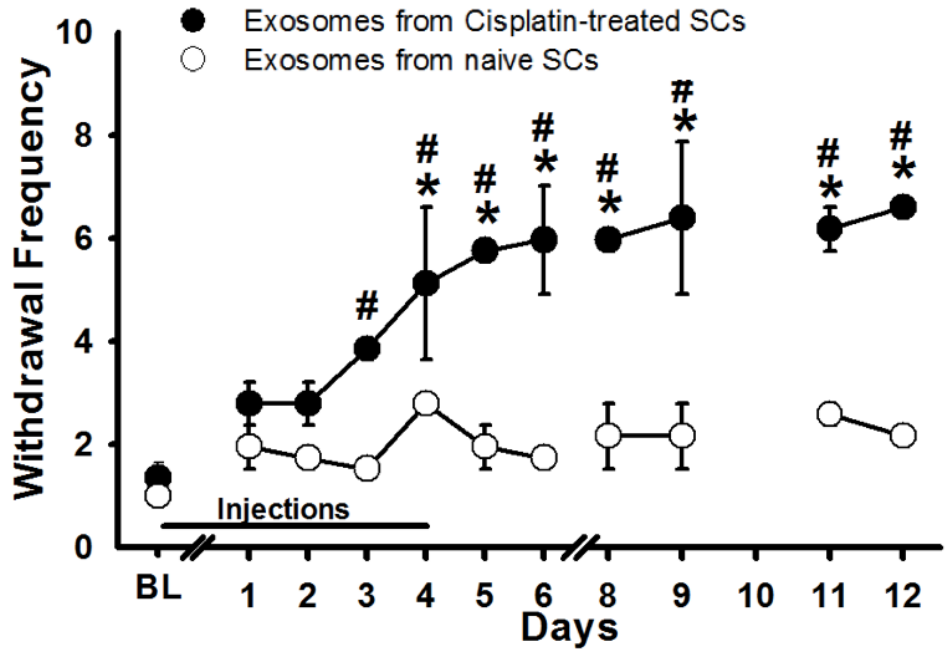


Figure 4.

A.


Exosomes from Cisplatin-treated SCs
Exosomes from naive SCs
Withdrawal Frequency
Injections
BL
Days

B.

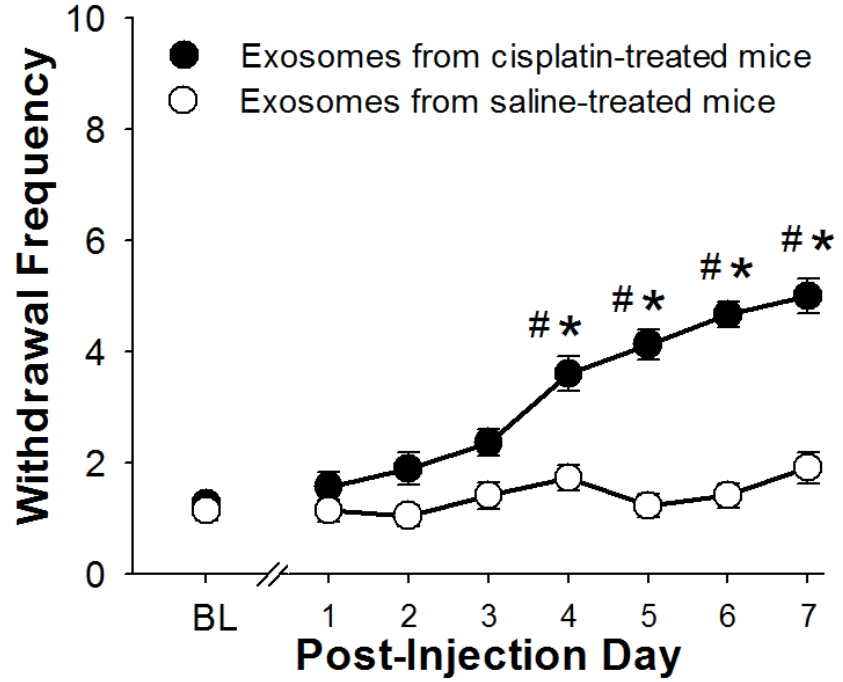
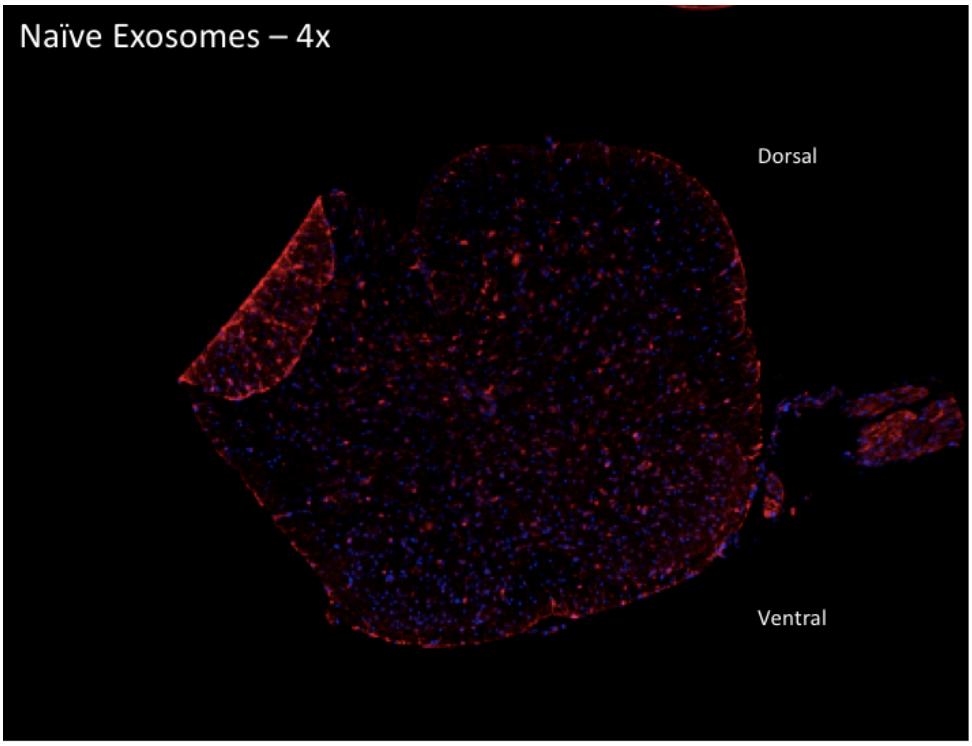
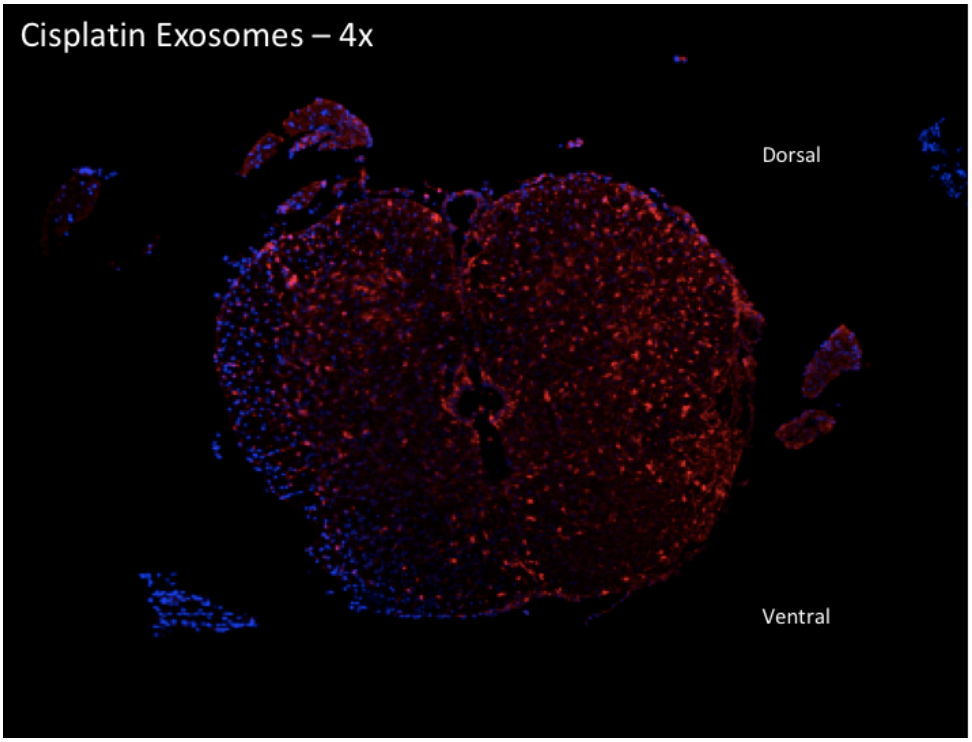

Exosomes from cisplatin-treated mice
Exosomes from saline-treated mice
Withdrawal Frequency
BL
Post-Injection Day

Figure 5.

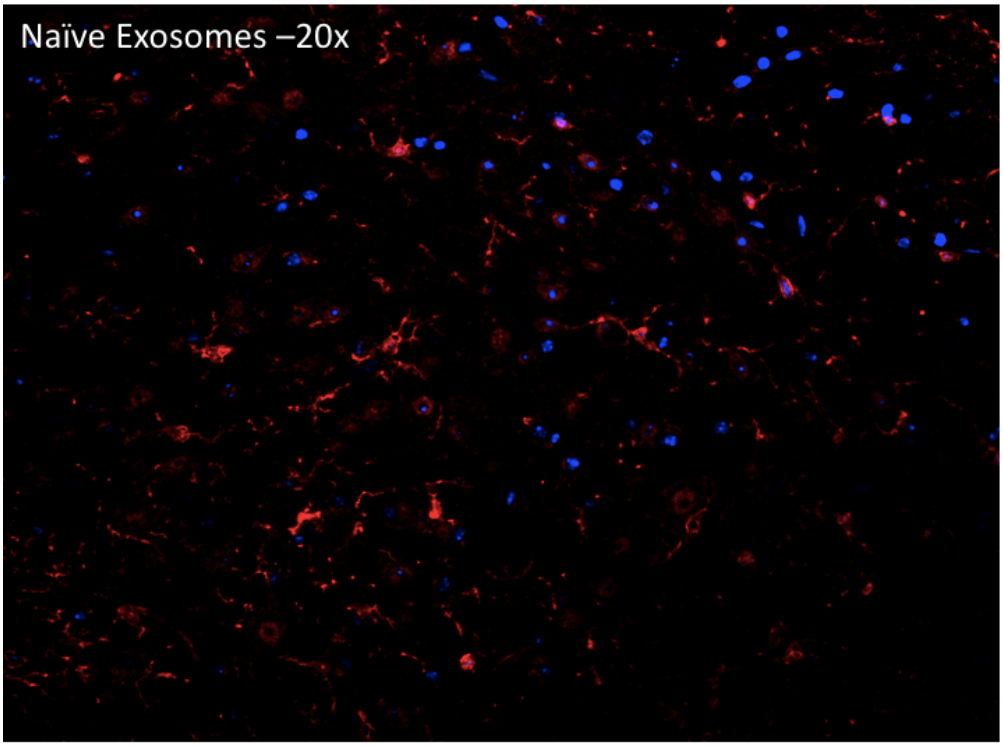
A.



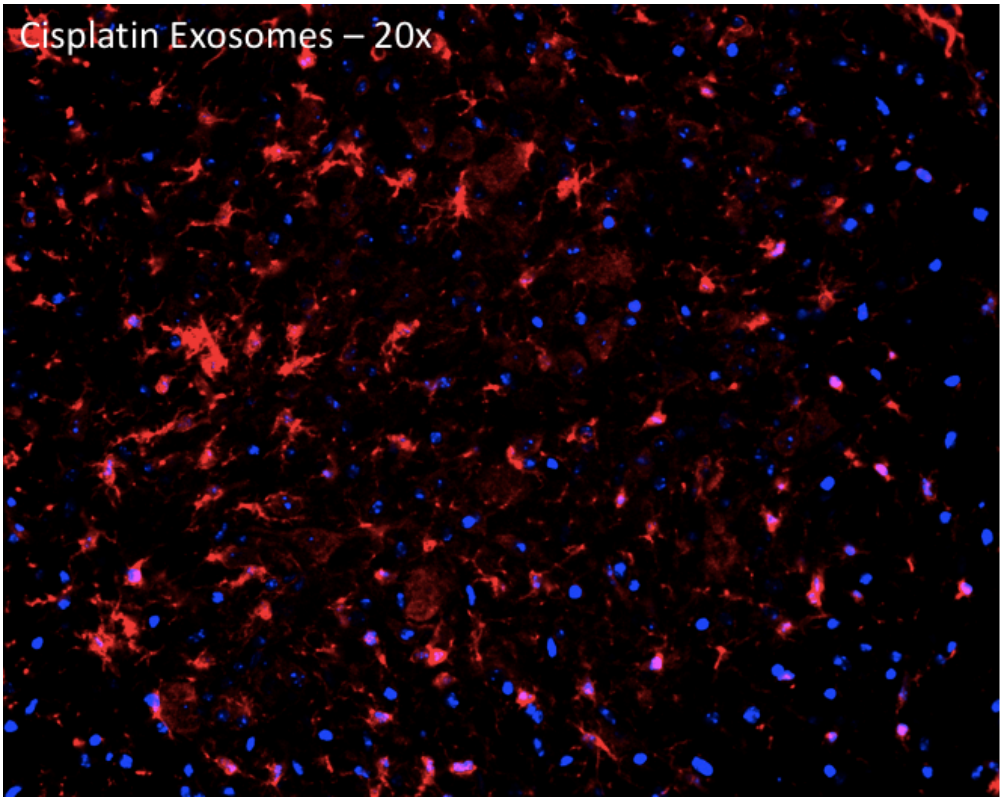
B.


Cisplatin Exosomes – 4x
Dorsal
Ventral

C.



D.


Cisplatin Exosomes – 20x

Bibliography

Alvarez, M. L., Khosroheidari, M., Ravi, R. K., & DiStefano, J. K. (2012). Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. Kidney international, 82(9), 1024-1032.

Araque, A., & Navarrete, M. (2010). Glial cells in neuronal network function. Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1551), 2375-2381.

Bahrini, I., Song, J. H., Diez, D., & Hanayama, R. (2015). Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia. Scientific reports, 5, 7989.

Basso, M., Pozzi, S., Tortarolo, M., Fiordaliso, F., Bisighini, C., Pasetto, L., ... & Bendotti, C. (2013). Mutant Copper-Zinc Superoxide Dismutase (SOD1) Induces Protein Secretion Pathway Alterations and Exosome Release in Astrocytes. Implications for Disease Spreading and Motor Neuron Pathology in Amyotrophic Lateral Sclerosis. Journal of Biological Chemistry, 288(22), 15699-15711.

Berta, T., Park, C. K., Xu, Z. Z., Xie, R. G., Liu, T., Lü, N., ... & Ji, R. R. (2014). Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-α secretion. The Journal of clinical investigation, 124(3), 1173-1186.

Blanden, M. J., Suazo, K. F., Hildebrandt, E. R., Hardgrove, D. S., Patel, M., Saunders, W. P., ... & Hougland, J. L. (2018). Efficient farnesylation of an extended C-terminal C (x) 3X sequence motif expands the scope of the prenylated proteome. Journal of Biological Chemistry, 293(8), 2770-2785.

Cancer Facts & Figures 2017(Rep.). (n.d.). Retrieved August 3, 2017, from American Cancer Society website: https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html

Carozzi, V. A., Canta, A., & Chiorazzi, A. (2015). Chemotherapy-induced peripheral neuropathy: What do we know about mechanisms?. Neuroscience letters, 596, 90-107.

Choi, D. S., Yang, J. S., Choi, E. J., Jang, S. C., Park, S., Kim, O. Y., ... & Gho, Y. S. (2012). The protein interaction network of extracellular vesicles derived from human colorectal cancer cells. Journal of proteome research, 11(2), 1144-1151.

Colombo, M., Raposo, G., & Théry, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual review of cell and

developmental biology, 30, 255-289.

Dasari, S., & Tchounwou, P. B. (2014). Cisplatin in cancer therapy: Molecular mechanisms of action. European Journal of Pharmacology,740, 364-378.

Dator, R., Carrà, A., Maertens, L., Guidolin, V., Villalta, P. W., & Balbo, S. (2017). A High Resolution/Accurate Mass (HRAM) Data-Dependent MS 3 Neutral Loss Screening, Classification, and Relative Quantitation Methodology for Carbonyl Compounds in Saliva. Journal of The American Society for Mass Spectrometry, 28(4), 608-618.

De Toro, J., Herschlik, L., Waldner, C., & Mongini, C. (2015). Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications. Frontiers in immunology, 6, 203.

Fields, R. D., & Stevens-Graham, B. (2002, October 18). New insights into neuron-glia communication. . Science, 298(5593), 556-562. Retrieved August 3, 2017, from https://www.ncbi.nlm.nih.gov/pubmed.

Florea, A., & Büsselberg, D. (2011). Cisplatin as an Anti-Tumor Drug: Cellular Mechanisms of Activity, Drug Resistance and Induced Side Effects. Cancers,3(4), 1351-1371. doi:10.3390/cancers3011351

Fröhlich, D., Kuo, W. P., Frühbeis, C., Sun, J. J., Zehendner, C. M., Luhmann, H. J., ... & Krämer-Albers, E. M. (2014). Multifaceted effects of oligodendroglial exosomes on neurons: impact on neuronal firing rate, signal transduction and gene regulation. Phil. Trans. R. Soc. B, 369(1652), 20130510.

Frühbeis, C., Fröhlich, D., & Krämer-Albers, E. M. (2012). Emerging roles of exosomes in neuron–glia communication. Frontiers in physiology, 3, 119.

Frühbeis, C., Fröhlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., ... & Schneider, A. (2013). Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte–neuron communication. PLoS biology, 11(7), e1001604.

Gercel-Taylor, C., Atay, S., Tullis, R. H., Kesimer, M., & Taylor, D. D. (2012). Nanoparticle analysis of circulating cell-derived vesicles in ovarian cancer patients. Analytical biochemistry, 428(1), 44-53.

Glenn, T. D., & Talbot, W. S. (2013). Signals regulating myelination in peripheral nerves and the Schwann cell response to injury. Current Opinion in Neurobiology,23(6), 1041-1048. doi:10.1016/j.conb.2013.06.010

Gregg, R. W., Molepo, J. M., Monpetit, V. J., Mikael, N. Z., Redmond, D., Gadia, M., & Stewart, D. J. (1992). Cisplatin neurotoxicity: the relationship between dosage, time, and

platinum concentration in neurologic tissues, and morphologic evidence of toxicity. Journal of Clinical Oncology,10(5), 795-803.

Grisold, W., Oberndorfer, S., & Windebank, A. J. (2012). Chemotherapy and Polyneuropathies. European Association of NeuroOncology Magazine,2(1), 25-36.

Guo, L., & Guo, N. (2015). Exosomes: Potent regulators of tumor malignancy and potential bio-tools in clinical application. Critical reviews in oncology/hematology, 95(3), 346-358.

Gupta, A., & Pulliam, L. (2014). Exosomes as mediators of neuroinflammation. Journal of neuroinflammation, 11(1), 68.

Helwa, I., Cai, J., Drewry, M. D., Zimmerman, A., Dinkins, M. B., Khaled, M. L., ... & Hamrick, M. W. (2017). A comparative study of serum exosome isolation using differential ultracentrifugation and three commercial reagents. PloS one, 12(1), e0170628.

Hylden, J. L., & Wilcox, G. L. (1980). Intrathecal morphine in mice: a new technique. European journal of pharmacology, 67(2-3), 313-316.

Ji, R. R., Berta, T., & Nedergaard, M. (2013). Glia and pain: is chronic pain a gliopathy?. Pain, 154, S10-S28.

Joseph, E. K., & Levine, J. D. (2009). Comparison of oxaliplatin-and cisplatin-induced painful peripheral neuropathy in the rat. The Journal of Pain, 10(5), 534-541.

Kalani, A., Tyagi, A., & Tyagi, N. (2013). Exosomes: Mediators of Neurodegeneration, Neuroprotection and Therapeutics. Molecular Neurobiology,49(1), 590-600. doi:10.1007/s12035-013-8544-1

Khasabova, I. A., Khasabov, S., Paz, J., Harding-Rose, C., Simone, D. A., & Seybold, V. S. (2012). Cannabinoid type-1 receptor reduces pain and neurotoxicity produced by chemotherapy. Journal of Neuroscience, 32(20), 7091-7101.

Khasabova, I. A., Holman, M., Morse, T., Burlakova, N., Coicou, L., Harding-Rose, C., . . . Seybold, V. S. (2013). Increased anandamide uptake by sensory neurons contributes to hyperalgesia in a model of cancer pain. Neurobiology of Disease,58, 19-28.

Kowal, J., Tkach, M., & Théry, C. (2014). Biogenesis and secretion of exosomes. Current opinion in cell biology, 29, 116-125.

Kuno, R., Wang, J., Kawanokuchi, J., Takeuchi, H., Mizuno, T., & Suzumura, A. (2005). Autocrine activation of microglia by tumor necrosis factor-α. Journal of neuroimmunology, 162(1), 89-96.

Kuo, C. C., Su, H. L., Chang, T. L., Chiang, C. Y., Sheu, M. L., Cheng, F. C., ... & Pan, H. C. (2017). Prevention of axonal degeneration by perineurium injection of mitochondria in a sciatic nerve crush injury model. Neurosurgery, 80(3), 475-488.

Lagunas, V.M., & Meléndez-Zajgla, J. (2008). Nuclear Factor-kappa B as a resistance factor to platinum-based antineoplasic drugs. Metal-based drugs, 2008.

Lankford, K. L., Arroyo, E. J., Nazimek, K., Bryniarski, K., Askenase, P. W., & Kocsis, J. D. (2018). Intravenously delivered mesenchymal stem cell-derived exosomes target M2-type macrophages in the injured spinal cord. PloS one, 13(1), e0190358.
Lawrence, T. (2009). The nuclear factor NF-κB pathway in inflammation. Cold Spring Harbor perspectives in biology, 1(6), a001651.

Lewitus, G. M., Konefal, S. C., Greenhalgh, A. D., Pribiag, H., Augereau, K., & Stellwagen, D. (2016). Microglial TNF-α suppresses cocaine-induced plasticity and behavioral sensitization. Neuron, 90(3), 483-491.

Lopez-Verrilli, M. A., Picou, F., & Court, F. A. (2013). Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. Glia,61(11), 1795-1806.

Martindale, J. L., & Holbrook, N. J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. Journal of Cellular Physiology,192(1), 1-15.

McDonald, M. K., Tian, Y., Qureshi, R. A., Gormley, M., Ertel, A., Gao, R., ... & Ajit, S. K. (2014). Functional significance of macrophage-derived exosomes in inflammation and pain. Pain®, 155(8), 1527-1539.

Menay, F., Herschlik, L., De Toro, J., Cocozza, F., Tsacalian, R., Gravisaco, M. J., ... & Mongini, C. (2017). Exosomes isolated from ascites of T-cell lymphoma-bearing mice expressing surface CD24 and HSP-90 induce a tumor-specific immune response. Frontiers in immunology, 8, 286.

Murphy, M. P., & LeVine III, H. (2010). Alzheimer's disease and the amyloid-β peptide. Journal of Alzheimer's Disease, 19(1), 311-323.

Olson, J. K. (2010). Immune response by microglia in the spinal cord. Annals of the New York Academy of Sciences, 1198(1), 271-278.

Park, H. J., Stokes, J. A., Corr, M., & Yaksh, T. L. (2014). Toll-like receptor signaling regulates cisplatin-induced mechanical allodynia in mice. Cancer chemotherapy and pharmacology, 73(1), 25-34.

Pegtel, D. M., Cosmopoulos, K., Thorley-Lawson, D. A., van Eijndhoven, M. A., Hopmans, E. S., Lindenberg, J. L., ... & Middeldorp, J. M. (2010). Functional delivery of viral miRNAs via exosomes. Proceedings of the National Academy of Sciences, 107(14), 6328-6333.

Podratz, J. L., Knight, A. M., Ta, L. E., Staff, N. P., Gass, J. M., Genelin, K., . . . Windebank, A. J. (2011). Cisplatin induced Mitochondrial DNA damage in dorsal root ganglion neurons. Neurobiology of Disease,41(3), 661-668.

Prinz, M., & Priller, J. (2014). Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. Nature Reviews Neuroscience, 15(5), 300.

Quintes, S., Goebbels, S., Saher, G., Schwab, M. H., & Nave, K. (2010). Neuron-glia signaling and the protection of axon function by Schwann cells. Journal of the Peripheral Nervous System,15(1), 10-16. doi:10.1111/j.1529-8027.2010.00247.

Rajendran, L., Honsho, M., Zahn, T. R., Keller, P., Geiger, K. D., Verkade, P., & Simons, K. (2006). Alzheimer's disease β-amyloid peptides are released in association with exosomes. Proceedings of the National Academy of Sciences, 103(30), 11172-11177.

Santangelo, L., Battistelli, C., Montaldo, C., Citarella, F., Strippoli, R., & Cicchini, C. (2017). Functional roles and therapeutic applications of exosomes in hepatocellular carcinoma. BioMed research international, 2017.

Streckmann, F., Balke, M., Lehmann, H. C., Rustler, V., Koliamitra, C., Elter, T., ... & Baumann, F. T. (2018). The preventive effect of sensorimotor-and vibration exercises on the onset of Oxaliplatin-or vinca-alkaloid induced peripheral neuropathies-STOP. BMC cancer, 18(1), 62.

Tang, Y. T., Huang, Y. Y., Zheng, L., Qin, S. H., Xu, X. P., An, T. X., ... & Wang, Q. (2017). Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. International journal of molecular medicine, 40(3), 834-844.

Tran, H., Gupta, M., & Gupta, K. (2017). Targeting novel mechanisms of pain in sickle cell disease. Blood, 130(22), 2377-2385.

Travis, L. B., Fossa, S. D., Sesso, H. D., Frisina, R. D., Herrmann, D. N., Beard, C. J., ... & Einhorn, L. H. (2014). Chemotherapy-induced peripheral neurotoxicity and ototoxicity: new paradigms for translational genomics. JNCI: Journal of the National Cancer Institute, 106(5).

Tsuda, M. (2017). Modulation of Pain and Itch by Spinal Glia. Neuroscience bulletin, 1-8.

Uhelski, M. L., Khasabova, I. A., & Simone, D. A. (2015). Inhibition of anandamide hydrolysis attenuates nociceptor sensitization in a murine model of chemotherapy-induced peripheral neuropathy. Journal of Neurophysiology, 113(5), 1501–1510.

Vlassov, A. V., Magdaleno, S., Setterquist, R., & Conrad, R. (2012). Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochimica et Biophysica Acta (BBA)-General Subjects, 1820(7), 940-948.

Willms, E., Johansson, H. J., Mäger, I., Lee, Y., Blomberg, K. E. M., Sadik, M., ... & Wood, M. J. (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. Scientific reports, 6, 22519.

Xiao, W. H., Zheng, H., Zheng, F. Y., Nuydens, R., Meert, T. F., & Bennett, G. J. (2011). Mitochondrial abnormality in sensory, but not motor, axons in paclitaxel-evoked painful peripheral neuropathy in the rat. Neuroscience, 199, 461-469.